

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**A transgene-assisted genetic screen identifies essential regulators of vascular development in vertebrate embryos.**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/92862> since

*Publisher:*

Academic Press Incorporated:6277 Sea Harbor Drive:Orlando, FL 32887:(800)543-9534, (407)345-4100,

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

## A transgene-assisted genetic screen identifies essential regulators of vascular development in vertebrate embryos

Suk-Won Jin<sup>a,\*</sup>, Wiebke Herzog<sup>a,\*</sup>, Massimo M. Santoro<sup>a,c</sup>, Tracy S. Mitchell<sup>a,3</sup>, Julie Frantsve<sup>a</sup>, Benno Jungblut<sup>a</sup>, Dimitris Beis<sup>a,4</sup>, Ian C. Scott<sup>a,5</sup>, Leonard A. D'Amico<sup>a</sup>, Elke A. Ober<sup>a,6</sup>, Heather Verkade<sup>a,7</sup>, Holly A. Field<sup>a,8</sup>, Neil C. Chi<sup>a</sup>, Ann M. Wehman<sup>b</sup>, Herwig Baier<sup>b</sup>, Didier Y. R. Stainier<sup>a,\*</sup>

<sup>a</sup> Department of Biochemistry and Biophysics, Programs in Developmental Biology, Genetics and Human Genetics, and Cardiovascular Research Institute, University of California San Francisco, 1550 Fourth Street, San Francisco, CA 94158, USA

<sup>b</sup> Department of Physiology, Neuroscience Program, University of California San Francisco, 1550 Fourth Street, San Francisco, CA 94158, USA

<sup>c</sup> Department of Environmental and Life Sciences, University of Piemonte Orientale "A. Avogadro", Italy

Received for publication 30 January 2007; revised 30 March 2007; accepted 30 March 2007

Available online 5 April 2007

### Abstract

Formation of a functional vasculature during mammalian development is essential for embryonic survival. In addition, imbalance in blood vessel growth contributes to the pathogenesis of numerous disorders. Most of our understanding of vascular development and blood vessel growth comes from investigating the Vegf signaling pathway as well as the recent observation that molecules involved in axon guidance also regulate vascular patterning. In order to take an unbiased, yet focused, approach to identify novel genes regulating vascular development, we performed a three-step ENU mutagenesis screen in zebrafish. We first screened live embryos visually, evaluating blood flow in the main trunk vessels, which form by vasculogenesis, and the intersomitic vessels, which form by angiogenesis. Embryos that displayed reduced or absent circulation were fixed and stained for endogenous alkaline phosphatase activity to reveal blood vessel morphology. All putative mutants were then crossed into the *Tg(flk1:EGFP)<sup>s843</sup>* transgenic background to facilitate detailed examination of endothelial cells in live and fixed embryos.

We screened 4015 genomes and identified 30 mutations affecting various aspects of vascular development. Specifically, we identified 3 genes (or loci) that regulate the specification and/or differentiation of endothelial cells, 8 genes that regulate vascular tube and lumen formation, 8 genes that regulate vascular patterning, and 11 genes that regulate vascular remodeling, integrity and maintenance. Only 4 of these genes had previously been associated with vascular development in zebrafish illustrating the value of this focused screen. The analysis of the newly defined loci should lead to a greater understanding of vascular development and possibly provide new drug targets to treat the numerous pathologies associated with dysregulated blood vessel growth.

© 2007 Elsevier Inc. All rights reserved.

**Keywords:** Zebrafish; Mutagenesis; Vascular development

\* Corresponding authors.

E-mail addresses: [suk-won.jin@med.unc.edu](mailto:suk-won.jin@med.unc.edu) (S.-W. Jin), [wiebke.herzog@ucsf.edu](mailto:wiebke.herzog@ucsf.edu) (W. Herzog), [didier\\_stainier@biochem.ucsf.edu](mailto:didier_stainier@biochem.ucsf.edu) (D.Y.R. Stainier).

<sup>1</sup> These authors contributed equally.

<sup>2</sup> Current address: Carolina Cardiovascular Biology Center and Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, NC 27599, USA.

<sup>3</sup> Current address: Osteoarthritis Research Group, Wyeth Research, 200 Cambridge Park Drive, Cambridge, MA 02140, USA.

<sup>4</sup> Current address: Foundation for Biomedical Research of the Academy of Athens, Basic Research Center, Athens, Greece.

<sup>5</sup> Current address: The Hospital for Sick Children, University of Toronto, 555 University Ave. Toronto, ONT, Canada M5G1X8.

<sup>6</sup> Current address: National Institute for Medical Research, Division of Developmental Biology, Mill Hill, London NW7 1AA, UK.

<sup>7</sup> Current address: School of Biological Sciences, Monash University, Clayton VIC 3800, Australia.

<sup>8</sup> Current address: Novartis Institutes for BioMedical Research, Inc. 250 Massachusetts Ave., Cambridge, MA 02139, USA.

## Introduction

The vasculature needs to undergo continuous modification and remodeling to accommodate the diverse needs for growth, regeneration, and repair during an organism's life. This dynamic modulation of the vascular system is achieved by intricate interactions between two distinct mechanisms, vasculogenesis (*de novo* assembly of vessels) and angiogenesis (modification and expansion of pre-existing vessels) (reviewed by Risau, 1997; Risau and Flamme, 1995).

Failure to regulate vasculogenesis and angiogenesis has been implicated in a wide variety of pathological conditions. Excessive vascular formation is usually associated with cancer, psoriasis, arthritis, and blindness, while insufficient vascular formation is involved in a variety of inherited diseases, as well as heart and brain ischemia, neurodegeneration, and osteoporosis (reviewed by Carmeliet, 2003, 2005; Carmeliet and Jain, 2000). The pathological consequences of dysregulated vascular formation have provided the impetus to understand the underlying principles of vascular system development and function, resulting in the identification of various signaling pathways and their downstream effectors (reviewed by Rossant and Howard, 2002).

The earliest event in this developmental cascade is the specification of endothelial cells, the cells lining all blood vessels. Initially, factors such as Bone Morphogenic Proteins (BMPs) (Gupta et al., 2006; Park et al., 2006) and Wnts (Lindsley et al., 2006; Wang et al., 2006) appear to define the number of potential endothelial progenitors within the nascent mesoderm. Subsequently Sonic Hedgehog (SHH) (Lawson et al., 2002; Vokes et al., 2004), Vascular Endothelial Growth Factor (VEGF) (Carmeliet et al., 1996; Ferrara et al., 1996; Cleaver and Krieg, 1998), and Notch (Krebs et al., 2000; Lawson et al., 2001) signaling pathways, as well as transcription factors including Ets family members (Dube et al., 1999; Sumanas and Lin, 2006; Pham et al., 2007), Scl/Tal1 (Kallianpur et al., 1994; Visvader et al., 1998; Patterson et al., 2005), and Coup-TFII (You et al., 2005) play critical roles in the differentiation of endothelial progenitors into arterial and venous endothelial cells. Endothelial cells then migrate towards the midline to form an aggregate known as the vascular cord, which subsequently lumenizes to form functional blood vessels (Torres-Vazquez et al., 2003; Jin et al., 2005). Extracellular matrix proteins such as Fibronectin, and mediators of cell movements such as Rac and Cdc42 provide critical functions during the migration of endothelial cells and vascular lumen formation (Jiang et al., 1994; Wijelath et al., 2002; Kamei et al., 2006). Nascent vascular networks then recruit vascular smooth muscle cells and pericytes, a process that requires the function of Platelet Derived Growth Factor (PDGF) signaling and EphrinB2–EphB4 interaction (Jain, 2003; Betsholtz et al., 2005).

Despite the progress in identifying some of the key factors required for vascular development, the functions of many signaling pathways and the interactions between them are poorly understood due to technical limitations of commonly used model systems. It is technically challenging to study the

mechanisms of vascular development since the intricate architecture and context of vascular networks are difficult to reproduce *in vitro* and the development of the vascular system is strongly influenced by interactions between vessels and neighboring tissues. In addition, inaccessibility of mammalian embryos during development makes *in vivo* analyses of vascular formation a difficult task. Furthermore, the indispensable function of the placental vasculature during mammalian development constitutes another obstacle for studying the effect of genetic mutations within the developing embryo.

To understand the fundamental principles of vascular development and identify essential genes in this process, we chose to utilize the zebrafish system, since it offers a unique opportunity to overcome the aforementioned technical difficulties. In addition to its well-documented amenability to forward genetic screens (Driever et al., 1996; Haffter et al., 1996; Amsterdam et al., 1999), the externally fertilized and optically clear embryos enable one to analyze the development of the vasculature *in vivo* at a cellular level. Recently generated vascular specific transgenic lines, such as *Tg(flk1:EGFP)<sup>s843</sup>* (Jin et al., 2005), facilitate the analyses. Although blood circulation starts at 24 hpf, zebrafish embryos can survive up to 7 dpf without a functional vasculature or heart beat (Stainier, 2001; Stainier et al., 1995; Sehnert et al., 2002), allowing one to study defects in vascular formation and patterning in the developing embryo over an extended period of time.

Here we present the results of a three-step forward genetic screen in zebrafish. Initially, we identified mutants with vascular defects by observing their blood circulation and subsequently stained them for endogenous alkaline phosphatase activity, which labels endothelial cells. These analyses were followed by an *in vivo* analysis using a transgenic endothelial specific GFP-reporter line (*Tg(flk1:EGFP)<sup>s843</sup>*).

We identified 30 distinct genetic loci that regulate vascular development. Mutations in 11 of these loci interfere with vasculogenesis by causing changes in the number of endothelial cells or in vascular tube and lumen formation. The other 19 mutations affect angiogenesis by disturbing vessel patterning, remodeling, integrity, and/or maintenance.

A majority of the identified mutations cause vascular defects in the context of otherwise unaffected embryos. Many of the vascular defects are similar to known human conditions such as hemangioma, aortic dissection, arterio-venous malformation, cerebral cavernous vascular malformation, and cerebral hemorrhage. Given the paucity of genetic models for these human vascular conditions, these zebrafish mutants should help understand the molecular and cellular etiology of these disorders as well as provide novel insights into vascular development.

## Materials and methods

### ENU mutagenesis and screening

Mutagenesis was performed by treating zebrafish (*Danio rerio*) males with the chemical mutagen *N*-nitroso-*N*-ethylurea, to induce mutations in premeiotic germ cells. Founder males were subjected to three to five treatments with ENU at weekly intervals and used to generate F2 generations, as previously described (Muto et al., 2005).

F3 embryos were first screened visually for defects in circulation to identify potential vascular mutants. Embryos with defective circulation were stained for endogenous alkaline phosphatase activity to highlight the vasculature. Embryos that showed absent or reduced endogenous alkaline phosphatase activity were further analyzed by *in situ* hybridization with *flkl* to determine the extent of their phenotype, and their parents were subsequently crossed into the *Tg(flkl:EGFP)<sup>s843</sup>* line (Jin et al., 2005) to analyze the phenotype at the cellular level. As a second transgenic line for phenotypic analysis we used *Tg(tie2:GFP)<sup>s849</sup>* (Motoike et al., 2000).

*Endogenous alkaline phosphatase activity assay, in situ hybridization, confocal analyses, immunohistochemistry, microangiography, and video microscopy*

Endogenous alkaline phosphatase activity assay was performed as previously described (Childs et al., 2002; Parker et al., 2004). Briefly, embryos were fixed in a 4% paraformaldehyde solution overnight and washed multiple times with 0.1% Triton in PBS, then stained in 10 mM Tris–HCl (pH 9). Alkaline phosphatase activity was detected by NBT/BCIP color reaction. *In situ* hybridizations were performed as previously described (Alexander and Stainier, 1999). Riboprobes for *flkl* (Liao et al., 1997), *ephrinB2a* (Chan et al., 2002; Lawson et al., 2001), *flt4* (Thompson et al., 1998), *tie2* (Lyons et al., 1998), *gata1* (Detrich et al., 1995), and *ve-cadherin* (Larson et al., 2004) were prepared with Roche Digoxigenin labeling kits. Embryos were mounted in benzylbenzoate:benzyl alcohol (2:1) and documented with a Zeiss Axiocam.

For confocal analyses, embryos were processed as previously described (Trinh and Stainier, 2004). Briefly, embryos were fixed overnight with 2% paraformaldehyde and embedded in 4% NuSieve GTG low melting agarose. Embedded embryos were cut with a VT1000S vibratome (Leica) into 250  $\mu$ m sections. Sections were processed in PBDT [1% BSA, 1% DMSO, and 0.1% Triton X-100 in PBS (pH 7.3)]. Mouse IgG anti- $\beta$ -catenin (Sigma) at 1:100 (Home-Badovinac et al., 2001) was used to detect endothelial cell boundaries, and TOPRO (Molecular Probes) at 1:10,000 (Oomman et al., 2004) was used to visualize nuclei. Processed samples were mounted in Vectashield (Vector Laboratories) and the images were acquired using a Zeiss LSM5 Pascal confocal microscope.

Microangiography was performed by injection of dextran-AlexaFluor594 (Invitrogen #D22913) into the common cardinal vein as previously described (Isogai et al., 2001). A Toshiba CCD video camera was used to capture the circulatory pattern of the embryos and Pinnacle Studio software was used to process the movies.

#### Complementation analyses and genetic mapping

Mutations were first categorized according to their phenotypes, and between mutations within the same group heterozygous individuals were crossed for complementation analyses. Mutations were subsequently mapped to linkage groups by bulk segregant analysis with SSLP markers.

## Results

### Genetic Screen for mutations that cause vascular defects

To identify genes with an essential function in vascular development, we used a forward genetic approach and performed a diploid F3 ENU mutagenesis screen in zebrafish. Specifically, the chemical mutagen ENU was used to induce premeiotic mutations in males which were subsequently bred to generate F2 carrier families as done in previous screens (Driever et al., 1996; Haffter et al., 1996).

F3 embryos were scored for reduced or absent circulation at either 36 or 60 hpf. Those with aberrant circulation pattern were fixed and stained at 36 hpf for endogenous alkaline phosphatase activity to visualize the presence and structure of

the vasculature. In order to maximize the specificity of the screen, embryos with vascular defects that are usually associated with hypoxia, such as tortuous subintestinal vessels (SIVs), were not kept for subsequent analyses (data not included). In a subset of potential mutants, *in situ* hybridization and confocal analyses were performed at various stages to define the time point when the vascular phenotype is first manifest. All recovered mutants were crossed to the *Tg(flkl:EGFP)<sup>s843</sup>* reporter line to analyze their vasculature in detail.

We screened a total of 2392 families, which, taking into account the analyzed number of families and offspring crosses per family (Mullins et al., 1994), can be calculated to correspond to 4015 mutagenized genomes. An extensive series of complementation tests and linkage group assignments indicate that these mutations define 30 different loci (Table 1).

According to the phenotype of the mutants and the onset of their vascular defects, we propose four distinct phases of vascular development: (I) specification and differentiation of endothelial precursors, (II) migration of these cells and subsequent vascular tube and lumen formation, (III) emergence and patterning of secondary vessels, and (IV) maintenance of vessels.

### Genes regulating the number of endothelial cells

To identify genes regulating the earlier steps of vascular development such as endothelial specification and/or differentiation, we performed a secondary *in situ* hybridization screen with embryos that displayed significant changes in the level of endogenous alkaline phosphatase activity, using the vascular specific marker *flkl* (Liao et al., 1997; Thompson et al., 1998). Three mutations, *mirinay<sup>s202</sup>* (*min*), *groom of cloche<sup>s635</sup>* (*grc*), and *santa<sup>s234</sup>* (*san*) show discernable changes in *flkl* expression (data not included). Further analyses with the *Tg(flkl:EGFP)<sup>s843</sup>* line confirmed that these mutations cause defects in early vascular development, most likely by affecting endothelial precursor formation (Figs. 1C and D; Fig. 2 and data not included). The number of endothelial cells in *min<sup>s202</sup>* and *grc<sup>s635</sup>* mutant embryos is drastically reduced (see Fig. S1), and appears to be comparable to that in the previously identified zebrafish mutant *cloche* (*clo*) (Fig. 1B), suggesting that the genes affected by these mutations are critical for endothelial specification and/or differentiation.

*min<sup>s202</sup>* mutant embryos show a significant reduction in the number of endothelial precursors during early development, most notably in the posterior portion of the embryos (Figs. 2D and H, S1). This phenotype becomes apparent at approximately 14 hpf. Surprisingly, these embryos form rudimentary axial vessels despite the initial deficit of endothelial cells. However, axial vessels in *min<sup>s202</sup>* mutant embryos appear to be discontinuous and do not form a proper lumen (Figs. 2I to L). Subsequent intersegmental vessel (SE) formation (Figs. 2I and J) and the specification of the arterial and venous endothelial cells also appear to be affected (Figs. 2N and P). In addition, homozygous *min<sup>s202</sup>* mutant embryos have a noticeable decrease in the number of primitive blood cells (Fig. 2R),



Table 1  
Classification of mutations identified in the screen

	Map position	Allele	Vascular defect	Other defects
<i>Group I mutations affecting the number of endothelial cells</i>				
<i>groom of cloche (grc)</i>	LG16	<i>s635</i>	Reduced number of endothelial cells	Heart defects
<i>mirinay (min)</i>	LG16	<i>s202</i>	Reduced number of endothelial cells	No blood cells
<i>santa (san)<sup>a</sup></i>	LG19	<i>s234</i>	Increased number of endothelial cells	See Stainier et al., 1996
<i>Group II mutations affecting vasculogenesis</i>				
<i>Group IIA mutations affecting the number of vascular lumens</i>				
<i>ménage a trois (mts)</i>	LG16	<i>s233</i>	Multiple lumens	Endoderm/fin defects
<i>solo (sol)</i>	LG14	<i>s828</i>	Defects in artery/vein segregation	
<i>you too (yot)<sup>a</sup></i>	LG9	<i>s406</i>	Defects in vascular cord formation	See Brand et al., 1996
<i>Group IIB mutations affecting the size of vascular lumens</i>				
<i>blind alley (bly)</i>	LG2	<i>s889</i>	Dilated posterior cardinal vein	
<i>poongsun (psn)</i>	LG15	<i>s634</i>	Dilated posterior cardinal vein	Blood regurgitation
<i>catacomb (ctb)</i>	N/D	<i>s479</i>	Dilated posterior cardinal vein	
<i>logelei (log)<sup>a</sup></i>	LG9	<i>s231</i>	Reduced endothelial cells/collapsed lumen	See Schier et al., 1996
<i>valentine (vtn)<sup>a</sup></i>	LG20	<i>s259</i>	Reduced common cardinal vein	See Stainier et al., 1996
<i>Group III mutations affecting vascular patterning</i>				
<i>disoriented (did)</i>	LG24	<i>s240</i>	Missing intersegmental vessels	
<i>cacophony (cpn)</i>	LG23	<i>s236</i>	Missing intersegmental vessels	
<i>quo vadis (qad)</i>	LG17	<i>s840</i>	Defects in brain vasculature	Smaller head
<i>unfinished (unf)</i>	LG23	<i>s808</i>	Defects in trunk and brain vasculature	Bent body
<i>way to go (way)</i>	LG5	<i>s409</i>	Defects in outflow tract and aortic arches	
<i>intersection (int)</i>	LG7	<i>s413</i>	Defects in dorsal aorta fusion	Heart/fin defects,
<i>sloppy vein (slv)</i>	N/D	<i>s887</i>	Defects in vein patterning	
<i>out of bounds (obd)<sup>a</sup></i>	LG8	<i>s601</i>	Sprouting of intersegmental vessels	See Chen et al., 2001
<i>Group IV mutations affecting vascular integrity/maintenance</i>				
<i>adrasteia (adr)</i>	LG 23	<i>s277</i>	Aortic arch dilation; later: circulation loop	
<i>losing grip (lgr)</i>	LG1	<i>s258</i>	Breakdown of arch vasculature	
<i>tomato (tom)</i>	LG21	<i>s805</i>	Apoptosis of endothelial cells	
<i>barolo (bar)</i>	LG8	<i>s847</i>	Apoptosis of endothelial cells	Cardiac edema
<i>wadi (wdi)</i>	N/D	<i>s631</i>	Breakdown of dorsal aorta	
<i>ruche (ruc)</i>	N/D	<i>s869</i>	Vascular regression	Big body
<i>stradivari (sra)</i>	N/D	<i>s877</i>	Vascular hemorrhage	
<i>reddish (reh)</i>	LG 8	<i>s587</i>	Vascular hemorrhage	
<i>gluten (gln)</i>	N/D	<i>s839</i>	Vascular regression	Smaller head
<i>nemo (nem)</i>	N/D	<i>s838</i>	Vascular regression	Cardiac edema
<i>violet beauregarde (vbg)<sup>a</sup></i>	LG23	<i>s407</i>	Defects in artery/vein segregation	See Roman et al., 2002

For all mutants identified in the screen, only one allele is shown.

<sup>a</sup> Indicates new allele of mutant identified in a previous screen.

suggesting that the gene affected by this mutation functions at a level prior to the specification of both lineages, as suggested for *clo* (Liao et al., 1997).

In contrast, *san*<sup>s234</sup> mutant embryos show an elevated expression level of endothelial specific markers (Fig. 3). Complementation analyses showed that the mutation we identified is allelic to a known mutation, *san*<sup>m775</sup>. Recently, positional cloning revealed that this mutation affects the *krit1/ccm1* gene (Mably et al., 2006), which has been implicated in cerebral cavernous malformation (Whitehead et al., 2004). However, the early vascular phenotype of *san/krit1* mutants has not been reported. In hemizygous *Tg(tie2:GFP)*<sup>s849</sup> embryos, the transgene is hard to detect due to its low expression level (Figs. 3G, I, and K). However, in *san*<sup>s234</sup> mutant embryos, the hemizygous *Tg(tie2:GFP)*<sup>s849</sup> transgene expression is significantly elevated, which results in its easy detection (Figs. 3H, J,

and L). Similarly, elevated levels of endogenous *tie2* expression were detected in *san*<sup>s234</sup> mutant embryos (Figs. 3D and F). Taken together, these observations suggest that *san/krit1* negatively regulates the expression level of endothelial specific genes and/or the number of endothelial cells. At later stages, *san*<sup>s234</sup> mutant embryos show a dilation of major vessels as observed in transgenic mice and human patients (Laberge-le Couteulx et al., 1999; Whitehead et al., 2004), which is most pronounced in the SIV (Fig. 3N) and the posterior cardinal vein (PCV), although the dorsal aorta (DA) appears relatively unaffected (Fig. 3P).

#### Genes regulating vascular tube or lumen structure

A subset of the mutants that display collapsed or dilated vessels were subjected to confocal microscopy analyses to test

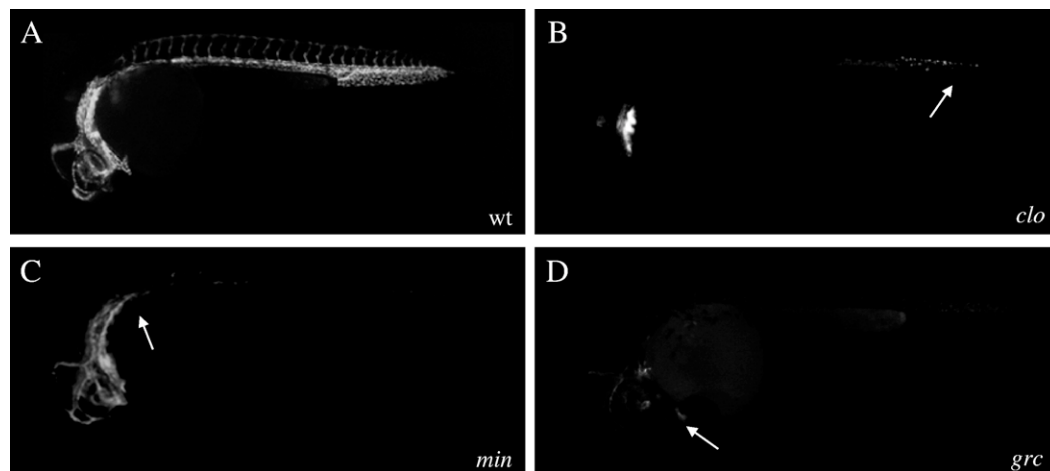


Fig. 1. Mutations affecting specification of endothelial precursors. (A–D) Epifluorescent micrographs of 36 hpf wild-type (A), *clo*<sup>s5</sup> mutant (B), *min*<sup>s202</sup> mutant (C), and *grc*<sup>s635</sup> mutant (D) embryos visualized by Tg(flk1:EGFP)<sup>s843</sup> expression. Morphologically, *grc*<sup>s635</sup> and *min*<sup>s202</sup> mutant embryos show no obvious defects, but the number of endothelial cells appears significantly reduced (C and D), a reduction comparable to that seen in *clo*<sup>s5</sup> mutant embryos (B). White arrows point to region with Tg(flk1:EGFP)<sup>s843</sup> positive endothelial cells are conspicuously missing.

whether these mutations affect vascular tube and lumen formation (Fig. 4). Through this analysis, we identified seven mutations that specifically affect vascular tube and lumen formation without affecting earlier steps of endothelial cell specification and differentiation (Table 1). These mutations can be subdivided into two groups depending on whether they affect the number (Group IIA) or the diameter (Group IIB) of the vascular lumen.

Two lumenized axial vessels, the DA and PCV, can be observed in wild-type embryos (Figs. 4A and B). The formation of the DA and PCV and subsequent lumenization of these vessels do not appear to be affected by lack of circulation, as shown in *silent heart/tnnt2* (*sih*) morpholino-injected embryos (Fig. 4C). Two mutations, *solo*<sup>s828</sup> (*sol*) and *ménage à trois*<sup>s233</sup> (*mts*), alter the number of lumens in axial vessels (Figs. 4D and E). In *sol*<sup>s828</sup> mutant embryos, endothelial cells fail to segregate into the DA and PCV, resulting in a single large vessel in the midline of the embryos (Fig. 4D). In contrast, an extra vascular lumen in the axial vessels is formed in homozygous *mts*<sup>s233</sup> mutant embryos (Fig. 4E). Further *in situ* analyses suggest that the *mts*<sup>s233</sup> mutation does not cause defects in the differentiation of endothelial cells into arterial or venous endothelial cells (Fig. S2).

Group IIB mutants, which show changes in the diameter of vascular lumen, include new alleles of two previously known mutations, *logelei* (*log*<sup>s231</sup>) (Schier et al., 1996) and *valentine* (*vt*<sup>s259</sup>) (Stainier et al., 1996) and three novel mutations, *poongsun*<sup>s634</sup> (*psn*), *blind alley*<sup>s889</sup> (*bly*), and *catacomb*<sup>s479</sup> (*ctb*). Axial vessels initially lumenize, but subsequently collapse in *log*<sup>s231</sup> mutant embryos (Fig. 4F), while the PCV fails to remodel and becomes grossly dilated in homozygous *psn*<sup>s634</sup>, *bly*<sup>s889</sup>, and *ctb*<sup>s479</sup> mutant embryos (Fig. 4G and data not included). However, we did not observe any obvious changes in the number or identity of endothelial cells in these mutants (Fig. S2 and data not included), suggesting that the molecular mechanisms that determine the size of vessels can be separated from those that regulate the specification of endothelial cells.

### Genes regulating vascular patterning

Formation of the complex vascular network requires vascular growth according to a pre-determined pattern. We identified eight mutants with specific defects in vascular patterning, all of which show wild-type like endothelial cell numbers, migration behavior, and axial vessel formation. Therefore, these mutations are likely to cause specific patterning defects as results of disturbed angiogenic processes rather than defective vasculogenesis. Four of the identified mutations affect early patterning of the SEs alone or in combination with other patterning events. Complementation analyses showed that we had identified multiple additional alleles of the previously identified *out of bounds* (*obd*<sup>s601</sup>) mutation (Chen et al., 2001; Childs et al., 2002), which displays an aberrant guidance of the SEs. In comparison, SEs in *disoriented*<sup>s240</sup> (*did*), *cacophony*<sup>s236</sup> (*cpn*), and *unfinished*<sup>s808</sup> (*unf*) mutant embryos, show distinct patterning defects: several SEs are missing in *did*<sup>s240</sup> and *cpn*<sup>s236</sup> mutant embryos, while partially formed SEs are observed in *unf*<sup>s808</sup> mutant embryos. Although the majority of SEs in *unf*<sup>s808</sup> mutant embryos are properly initiated, they fail to extend and contribute to the dorsal longitudinal anastomotic vessels (DLAVs) (Figs. 5D to F). In severely affected *unf*<sup>s808</sup> mutant embryos, SEs do not form at all (data not included). This failure of angiogenic sprouting also disturbs patterning of the head vasculature in *unf*<sup>s808</sup> mutant embryos. These head vascular defects are characterized by a failure in the sprouting from the primordial midbrain channel (PMBC) and the formation and extension of the hindbrain capillary network (data not included).

Another angiogenic process, the remodeling of the PCV, appears to be affected in embryos homozygous for the mutation *sloppy vein*<sup>s887</sup> (*slv*) (Figs. 5J to L). Although *slv*<sup>s887</sup> mutants also have minor aberrations in SE development (Fig. 5L), the overall patterning of the trunk and head vasculature appears to be unaffected.

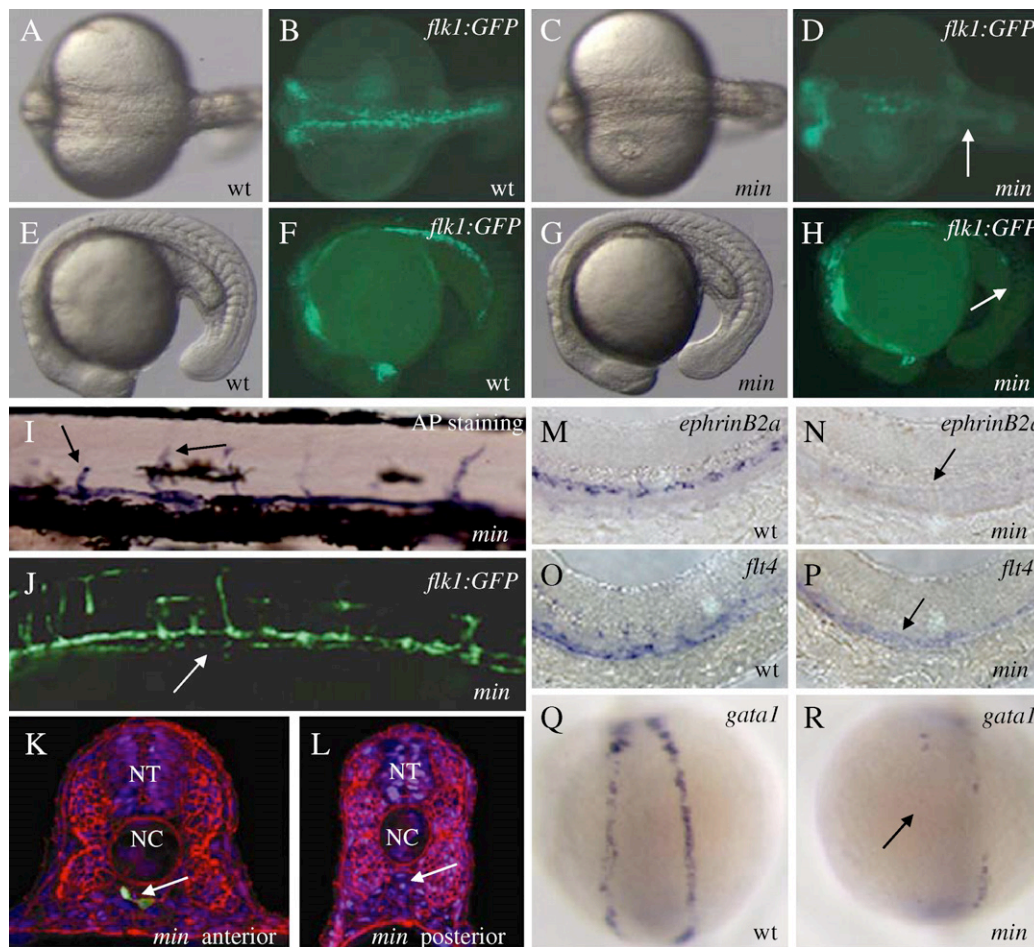


Fig. 2. *mirinay* regulates endothelial and hematopoietic lineages. (A–H) Bright-field (A, C, E, and G) and epifluorescent (B, D, F, and H) micrographs of 20 hpf wild-type (A, B, E, and F) and *min*<sup>s202</sup> mutant (C, D, G, and H) embryos, shown in dorsal (A to D) and lateral (E to H) views. Note the reduction of endothelial cells in the posterior region of *min*<sup>s202</sup> mutant embryos (arrows). (I–J) Micrographs of 96 hpf *min*<sup>s202</sup> mutant larvae stained for endogenous alkaline phosphatase activity (I, bright-field) and visualized for Tg(flk1:EGFP)<sup>s843</sup> expression (J). Black arrows point to arrested intersegmental vessels (SEs) (I), white arrow points to discontinuous axial vessel (J). (K, L) Transverse sections from anterior (K) and posterior (L) trunk of 36 hpf *min*<sup>s202</sup> mutant embryos, visualized for Tg(flk1:EGFP)<sup>s843</sup> expression (green),  $\beta$ -Catenin (red), and TOPRO (blue). Although the vasculature of *min*<sup>s202</sup> mutant embryos eventually recovers, a drastically reduced number of endothelial cells is observed at this stage (white arrows point to the region of axial vessels). (M–R) Defective endothelial cell specification in 24 hpf *min*<sup>s202</sup> mutant embryos (N and P) compared to wild-type embryos (M and O), as assessed by *in situ* hybridization with the arterial endothelial marker *ephrinB2a* (M and N), and the venous endothelial marker *flt4* (O and P); and defective erythropoiesis in 18 hpf *min*<sup>s202</sup> mutant embryos (R) compared to wild-type (Q), as assessed by examining *gata1* expression in dorsal views. Black arrows in panels N and P point to the reduction in endothelial marker expression in *min*<sup>s202</sup> mutant embryos, and black arrow in panel R points to the region of the lateral plate mesoderm where erythrocytes form in wild-type embryos. Abbreviations: NT: neural tube, NC: notochord.

The opposite seems to be the case for *quo vadis*<sup>s840</sup> (*qad*) mutant embryos. In *qad*<sup>s840</sup> mutants (Figs. 5Q to T), misguided patterning of the head vasculature, especially apparent in the hindbrain capillary network, can be observed. However, *qad*<sup>s840</sup> mutant embryos have no obvious patterning defects in their trunk and tail vasculature (Fig. 5R). The phenotype becomes apparent relatively late in development: at 48 hpf *qad*<sup>s840</sup> mutant embryos are indistinguishable from their siblings, but after 72 hpf mutants can be identified by their looping brain vessels (Figs. 5S and T) as well as smaller head and eyes. *way to go*<sup>s409</sup> (*way*) mutant embryos are characterized by a failure to form the arch vasculature, starting at 48 hpf, and also show no trunk or tail patterning defects (data not included).

All patterning mutants identified seem to affect angiogenic vascular growth, indicating that many different factors contribute to vessel outgrowth and patterning. However, in

*intersection*<sup>s413</sup> (*int*) mutants, specific disruption of patterning leads to a failure in connecting the two lateral dorsal aortae (LDA) into the single DA and a failure in fusing the anterior cardinal veins (ACVs) with their respective PCVs to form the common cardinal veins (CCVs) (Fig. 6). In addition PCV development is affected in *int*<sup>s413</sup> mutants (Fig. 6, inset in panel D). Since this phenotype can be observed at 24 hpf, it is not clear whether cell migration and vasculogenic processes are also dysregulated in *int*<sup>s413</sup> mutant embryos. Interestingly, *int*<sup>s413</sup> mutant embryos establish a network of endothelial cells (as seen by Tg(flk1:EGFP)<sup>s843</sup> expression, Fig. 6H) and express the vascular cell adhesion gene *ve-cadherin* (Fig. 6F), indicating that assembly, remodeling, or arterial versus venous specification rather than cell migration is affected. This observation and the confinement of the phenotype to two sites of arterial-venous remodeling led us to classify *int*<sup>s413</sup> as a patterning mutant.



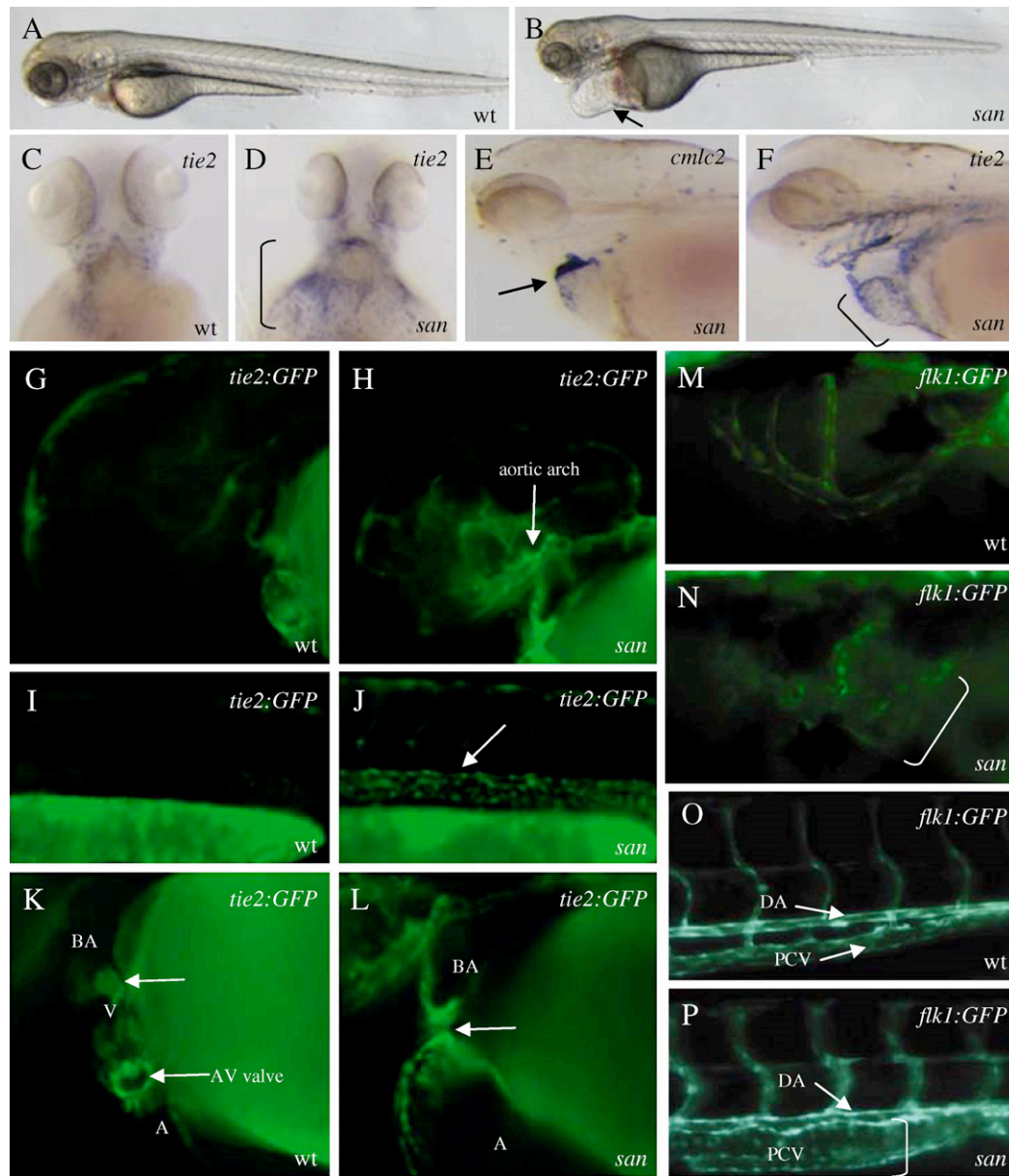


Fig. 3. *santa* negatively regulates vascular specific gene expression and endothelial cell morphology. (A–F) Bright-field micrographs of 60 hpf wild-type (A) and *san*<sup>s234</sup> mutant (B) larvae. Black arrow in panel B points to enlarged heart and pericardial cavity. Endothelial cells visualized by *in situ* hybridization with *tie2* in 60 hpf wild-type (C), and *san*<sup>s234</sup> mutant (D) larvae, shown in ventral view of the heart region. Black bracket in panel D demarcates the expansion of *tie2* expression in this region. Micrographs of *cmlc2* (E) and *tie2* (F) expression in 60 hpf *san* mutant larvae. The enlarged heart in *san*<sup>s234</sup> mutant larvae shows an apparent increase of *tie2* positive cells (black arrow in panel E and black bracket in panel F). (G–L) Epifluorescent micrographs of 36 hpf wild-type (G, I, and K) and *san* mutant (H, J, and L) *Tg(tie2:GFP)*<sup>s849</sup> embryos showing the aortic arches (G and H), trunk vessels (I and J), and heart (K and L). Note the elevated *Tg(tie2:GFP)*<sup>s849</sup> expression in *san*<sup>s234</sup> mutant embryos (white arrows). (M–P) Epifluorescent micrographs of 60 hpf wild-type (M and O) and *san*<sup>s234</sup> mutant (N and P) *Tg(flk1:EGFP)*<sup>s843</sup> larvae showing the subintestinal vessel (M and N) and posterior trunk (O and P). Vessels in *san*<sup>s234</sup> mutant larvae (white brackets) appear dilated compared to those in wild-type siblings. Abbreviations: BA: bulbus arteriosus, V: ventricle, A: atrium.

### Genes regulating vascular integrity

Mutants with defects in vascular integrity initially form and pattern the vascular network as their wild-type siblings, but are characterized by a subsequent partial or complete failure of circulation. Using the *Tg(flk1:EGFP)*<sup>s843</sup> line, we further characterized these mutants and confirmed that these genes regulate the maintenance or integrity of the vasculature.

We identified five novel mutations, including a new allele of the previously identified mutation *violet beauregarde* (*vbg*<sup>s407</sup>)

(Roman et al., 2002), that affect vascular maintenance due to the breakdown of specific vessels. Additionally, we identified two mutants *stradivari*<sup>s877</sup> (*sra*) and *reddish*<sup>s587</sup> (*reh*) with vascular hemorrhage, and three mutants *adrasteia*<sup>s277</sup> (*adr*), *barolo*<sup>s847</sup> (*bar*), and *tomato*<sup>s805</sup> (*tom*), which lose circulation gradually. In *losing grip*<sup>s258</sup> (*lgr*) mutant embryos, the breakdown of the vasculature can first be seen after 72 hpf and starts with the stretching or thinning out of the branchial arch vasculature, but the trunk and tail vasculature remain essentially unaffected (data not included).



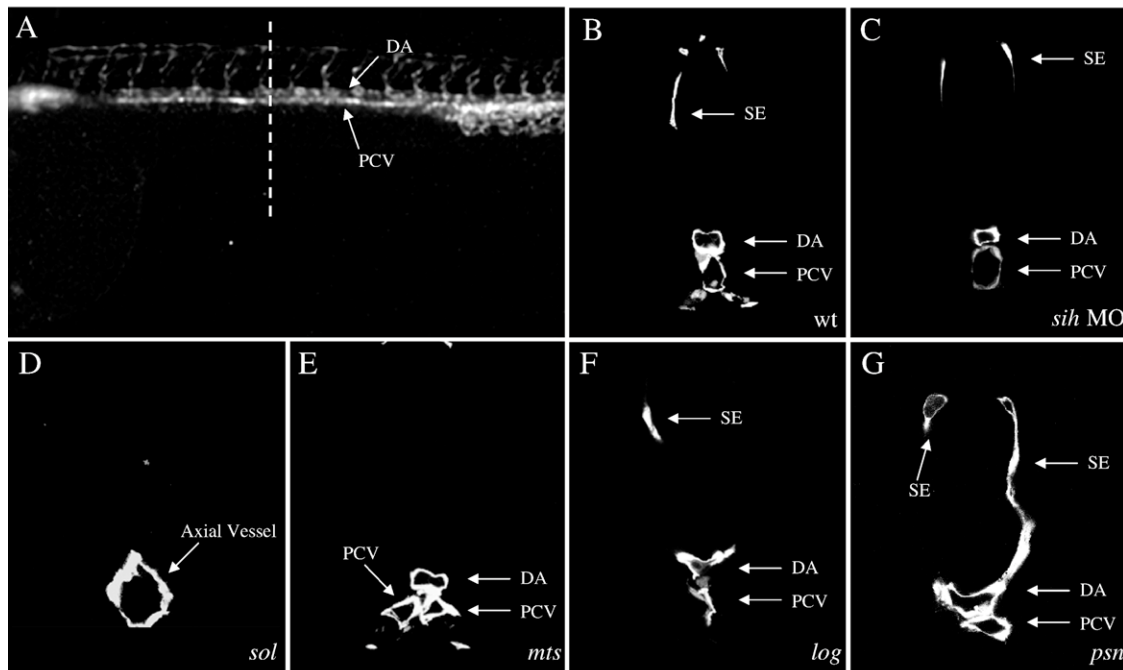


Fig. 4. Mutations affecting vascular tube formation. (A) Epifluorescent micrograph of 36 hpf *Tg(flk1:EGFP)*<sup>s843</sup> embryo in lateral view (A). White line marks the location of the transverse sections shown in panels B to F. (B–G) Transverse sections of wild-type (B), *sih/cmc2* MO injected (C), *sol*<sup>s828</sup> mutant (D), *mts*<sup>s233</sup> mutant (E), *log*<sup>s231</sup> mutant (F), and *psn*<sup>s634</sup> mutant (G) *Tg(flk1:EGFP)*<sup>s843</sup> embryos. Lack of circulation does not affect vascular lumen formation (C). Note changes in the number of vascular lumens in *sol*<sup>s828</sup> and *mts*<sup>s233</sup> mutant embryos, and in the size and shape of the vascular lumens in *log*<sup>s231</sup> and *psn*<sup>s634</sup> mutant embryos.

Similarly, the DA of homozygous *wadi*<sup>s631</sup> (*wdi*) mutant embryos starts to disintegrate at 72 hpf. However, homozygous *wdi*<sup>s631</sup> mutant embryos develop a circulation shunt, and are morphologically indistinguishable from their wild-type siblings under the light microscope (Figs. 7G and J, and data not included). Detailed observation with epifluorescent (Fig. 7L) and confocal microscopy (data not included) shows that the DA of *wdi*<sup>s631</sup> mutants regresses, and that circulation is rerouted to provide blood to the posterior region of the embryo.

In contrast, in *bar*<sup>s847</sup> mutant embryos, the vasculature degenerates much earlier. *bar* mutant embryos show significant global reduction of *Tg(flk1:EGFP)*<sup>s843</sup> expression in all endothelial cells starting at 48 hpf, and this reduction becomes more pronounced at 72 hpf (Figs. 7Q and R). Furthermore, the *bar*<sup>s847</sup> mutation causes severe vascular hemorrhage in the brain at 72 hpf, due to endothelial cell specific apoptosis (data not included). Surprisingly, these mutant embryos did not show any other major phenotype besides slight cardiac edema (Fig. 7P), indicating that this phenotype is caused by a disruption of a gene that functions within the endothelium and/or endocardium.

Whereas in most mutants of this group the vasculature disintegrates, collapses, or regresses in a mutant-specific way, in *adr*<sup>s277</sup> mutant embryos, we observed the opposite phenotype. A dilation of the head vasculature can first be seen in *adr*<sup>s277</sup> mutant embryos, most notably in the arch vessels, at 72 hpf (Figs. 7E and F). At this time point, circulation is still vigorous throughout the entire embryo. Within the following 48 h, circulation becomes progressively reduced, and through the formation of a shunt at 120 hpf blood flow is confined to a minimal loop through the heart (Fig. S3).

## Discussion

By using a combination of a fast initial and detailed transgenic line assisted secondary screen, we identified 30 distinct mutations from 4015 mutagenized genomes, that affect processes ranging from early vascular development, such as angioblast specification, to the later maintenance of the vasculature.

In this screen, we identified mutations that cause similar vascular defects to known human conditions, such as heman-gioma (in the case of *bly*<sup>s889</sup> and *psn*<sup>s634</sup>), aortic dissection (in case of *wdi*<sup>s631</sup>), arterio-venous malformations (in the case of *int*<sup>s413</sup> and *sol*<sup>s828</sup>), vascular hemorrhage (in the case of *sra*<sup>s887</sup> and *reh*<sup>s587</sup>), and cerebral cavernous vascular malformations (in the case of *san*<sup>s234</sup> and *vtn*<sup>s259</sup>). With the exception of *san* and *vtn*, the aforementioned mutations appear to be novel, a fact that validates our screening strategy and also supports the use of zebrafish for the identification of genes regulating the development and maintenance of the vasculature. In addition, the similarities between the phenotypes of newly identified zebrafish mutants and the symptoms of human vascular conditions indicate that future analyses of these mutants will provide invaluable insights towards understanding the etiology of various human vascular conditions.

Six loci identified in the screen (*san*, *vtn*, *log*, *vbg*, *yot*, and *obd*) had been identified in previous screens (Brand et al., 1996; Driever et al., 1996; Schier et al., 1996; Stainier et al., 1996; Chen et al., 2001; Roman et al., 2002). However, only *vbg*, *yot*, and *obd* (Roman et al., 2002; Lawson et al., 2002; Childs et al., 2002) had previously been implicated in vascular development. *san*, *vtn*, and *log* had been identified due to other

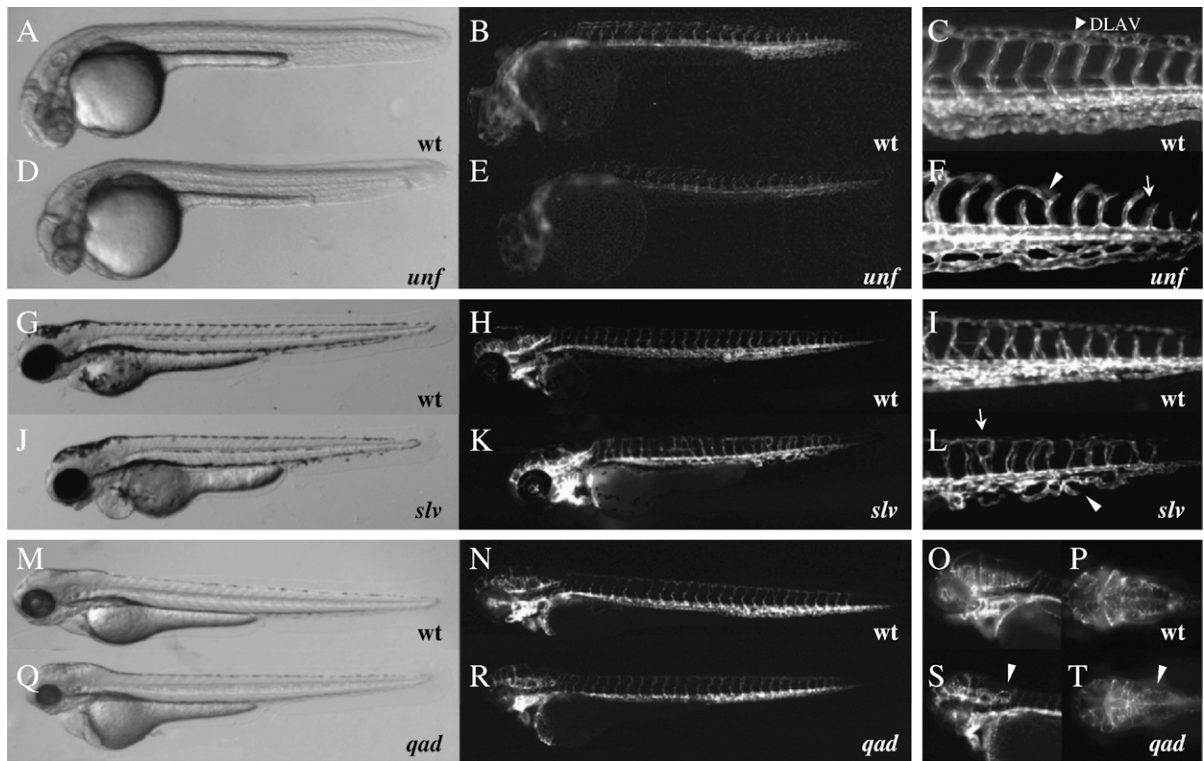


Fig. 5. Mutations affecting vascular patterning. (A–T) Bright-field and epifluorescent micrographs of *Tg(flk1:EGFP)<sup>s843</sup>unf<sup>s808</sup>* mutant embryos (D, E, 30 hpf; F, 48 hpf), *slv<sup>s887</sup>* mutant (J–L, 78 hpf) and *qad<sup>s840</sup>* mutant (Q–R, 78 hpf) larvae and their wild-type (wt) siblings. (A–F) *unf<sup>s808</sup>* mutants have shortened intersegmental vessels (SEs, arrow in panel F) and fail to form the dorsal longitudinal anastomotic vessel (DLAV, arrowhead in panels C and F) and appear to have reduced sprouting angiogenesis in the head (E). (G–L) Mis-patterning of the intersegmental vessels (SEs, arrow in panel L) and failure to remodel the posterior cardinal vein (PCV, arrowhead in L) in *slv<sup>s887</sup>* mutant larvae. (M–S) Loop formation in the hindbrain capillary network in *qad<sup>s840</sup>* mutant larvae (P (wt) and T (*qad<sup>s840</sup>*) are dorsal views focusing on the hindbrain capillary network). Instead of connecting lateral to medial as in wild-type (O, P), in *qad<sup>s840</sup>* mutant larvae capillaries connect back to their vessel of origin (arrowheads in panels S, T). Note that the tail vasculature in *qad<sup>s840</sup>* mutants (R) appears indistinguishable from wild-type (N). *qad<sup>s840</sup>* mutant embryos and their wild-type siblings were PTU treated to allow better visualization of the head vasculature.

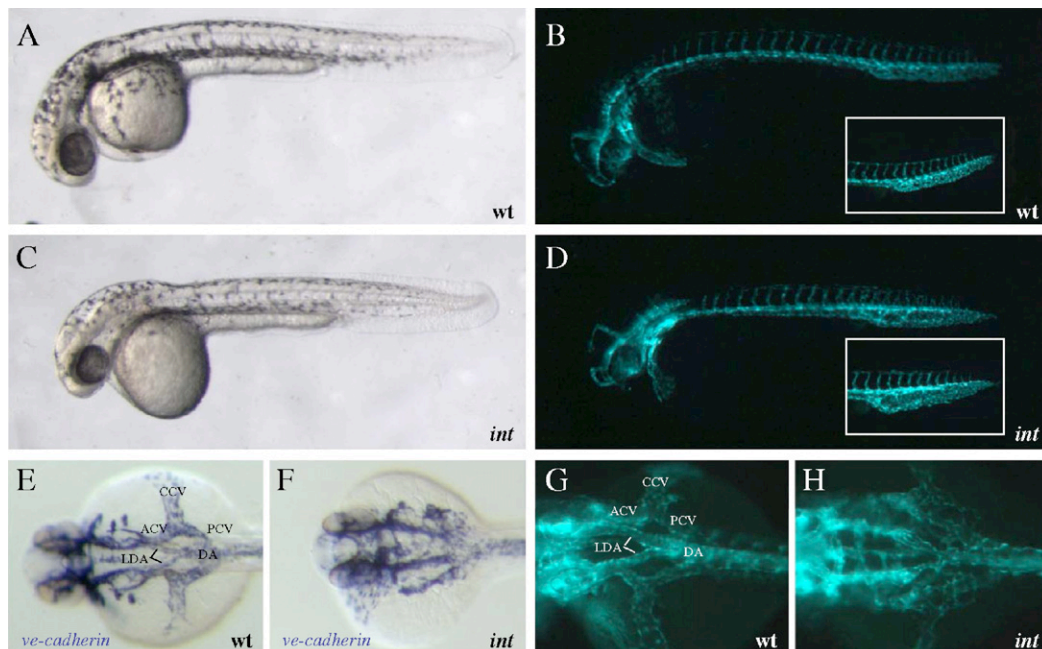


Fig. 6. *intersection* regulates vascular patterning. (A–D) Lateral bright-field and epifluorescent micrographs of 32 hpf embryos. Vascular patterning defects can be observed by visualizing *Tg(flk1:EGFP)<sup>s843</sup>* expression, but not via bright-field microscopy. *int<sup>s413</sup>* mutants fail to connect the lateral dorsal aortae (LDA) to form the dorsal aorta (DA), and the anterior and posterior cardinal veins (ACV and PCV) to the common cardinal vein (CCV). (E–H) Dorsal views of *ve-cadherin* and *Tg(flk1:EGFP)<sup>s843</sup>* expression. *int<sup>s413</sup>* mutant embryos exhibit a pronounced dilation of the PCV (inset in panel D).

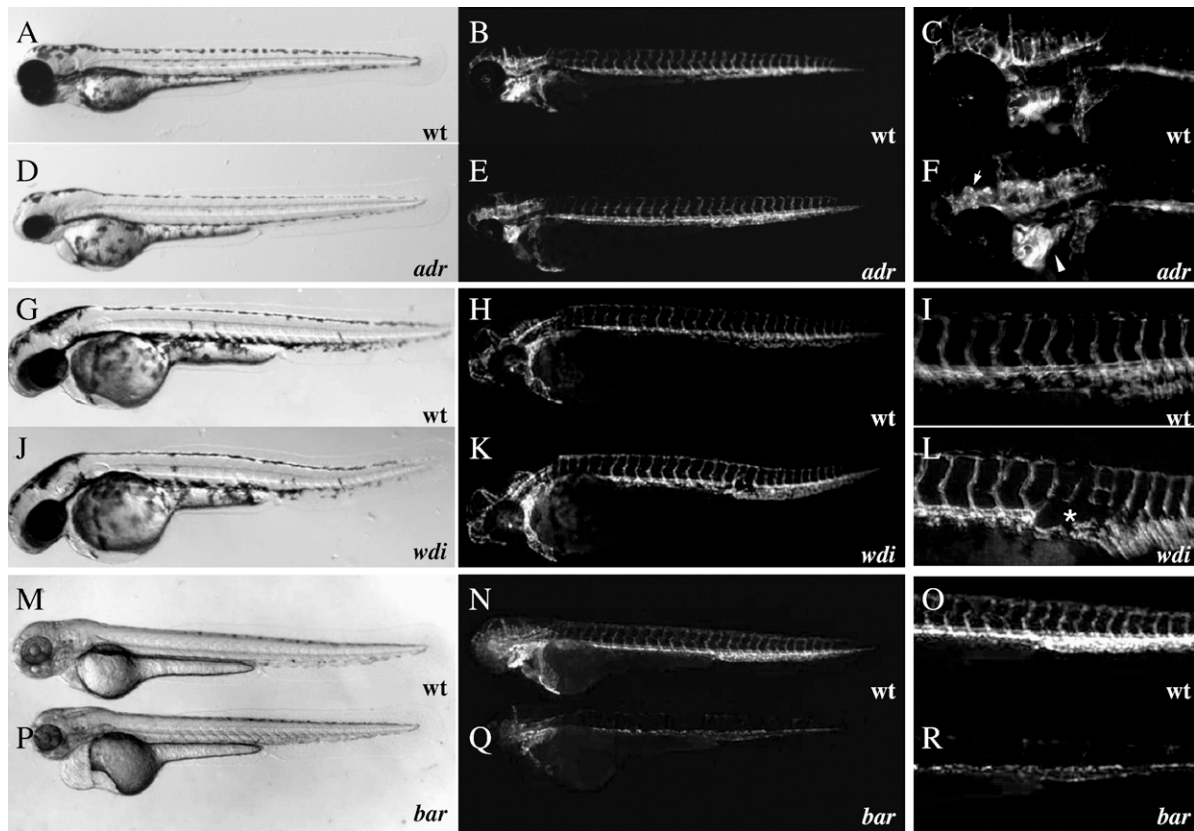


Fig. 7. Mutations affecting vascular maintenance and integrity. (A–R) Bright-field (A, D, G, J, M, and P) and epifluorescent (B, C, E, F, H, I, K, L, N, O, Q, and R) micrographs of *Tg(flk1:EGFP)<sup>s843</sup>adr<sup>s277</sup>* mutant larvae (D–F) and wild-type siblings (A–C), *wdi<sup>s631</sup>* mutant larvae (J–L) and wild-type siblings (G–I), and *bar<sup>s847</sup>* mutant larvae (P–R) and wild-type siblings (M–O) at 72 hpf. Affected areas within epifluorescent micrographs are magnified and shown in panels C, F, I, L, O, and R. Note the massive vascular dilation throughout the head vasculature in *adr<sup>s277</sup>* mutant larvae (F arrowheads), regression of the dorsal aorta in *wdi<sup>s631</sup>* mutant larvae (asterisk in panels I; K, L), and apparently disappearing endothelial cells in *bar<sup>s847</sup>* mutant larvae (Q, R).

developmental defects (cardiac phenotype in case of *san* and *vtn* (Stainier et al., 1996; Mably et al., 2006) and general body shape defects in case of *log* and *yot* (Schier et al., 1996; Brand et al., 1996)). Multiple alleles were identified for 7 loci. Specifically we identified 2 alleles each of *san*, *sol*, *log*, *vtn*, *adr*, and *vbg* as well as 5 alleles of *obd*. The identification of multiple alleles at certain loci may mean that they are mutagenic hot spots. Alternatively, it might reflect that a certain degree of saturation has been reached in conventional ENU based mutagenesis screens. Nevertheless, a majority of the mutations identified in our screen appear to be novel, suggesting that a modified screening method such as ours can identify new classes of mutants. Thus in order to favor the identification of novel loci, more sophisticated screens, such as transgene based ones or screens with a sensitized genetic background should be explored.

#### Use of transgenic fish as a new tool to identify subtle phenotypes

Several *in situ* based as well as vital staining based ENU mutagenesis screens have been performed to identify novel mutations affecting specific developmental processes (for example, Herzog et al., 2004). However, these secondary staining methods are usually time consuming and labor intensive. To identify mutations displaying subtle defects in

vascular development more quickly and effectively, we used a recently generated transgenic line, *Tg(flk1:EGFP)<sup>s843</sup>*, to visualize the developing vasculature, which enabled us to identify many mutations by simple observation of embryos under the epifluorescence microscope.

Although many mutants identified in our screen have additional morphological defects, several of them would not have been identified without the *Tg(flk1:EGFP)<sup>s843</sup>* line. As previously shown (Jin et al., 2005), GFP expression in the *Tg(flk1:EGFP)<sup>s843</sup>* line starts within endothelial progenitors at approximately 8 somites (13 hpf), and persists through adulthood. By employing the *Tg(flk1:EGFP)<sup>s843</sup>* line, we were able to identify mutants (e.g. *min<sup>s202</sup>*) that show defects in early angioblast specification but recover later during development. A similar transgene-assisted screening approach identified multiple novel mutations affecting endodermal organ development (Ober et al., 2006) and a novel gene affecting vascular patterning (Pham et al., 2007).

#### Distinct phases of vascular development revealed by distinct phenotypes

The phenotypes of the identified mutants reflect distinct phases of vascular development (Table 1 and Fig. 8): specification and migration of angioblasts, formation of vascular tubes,



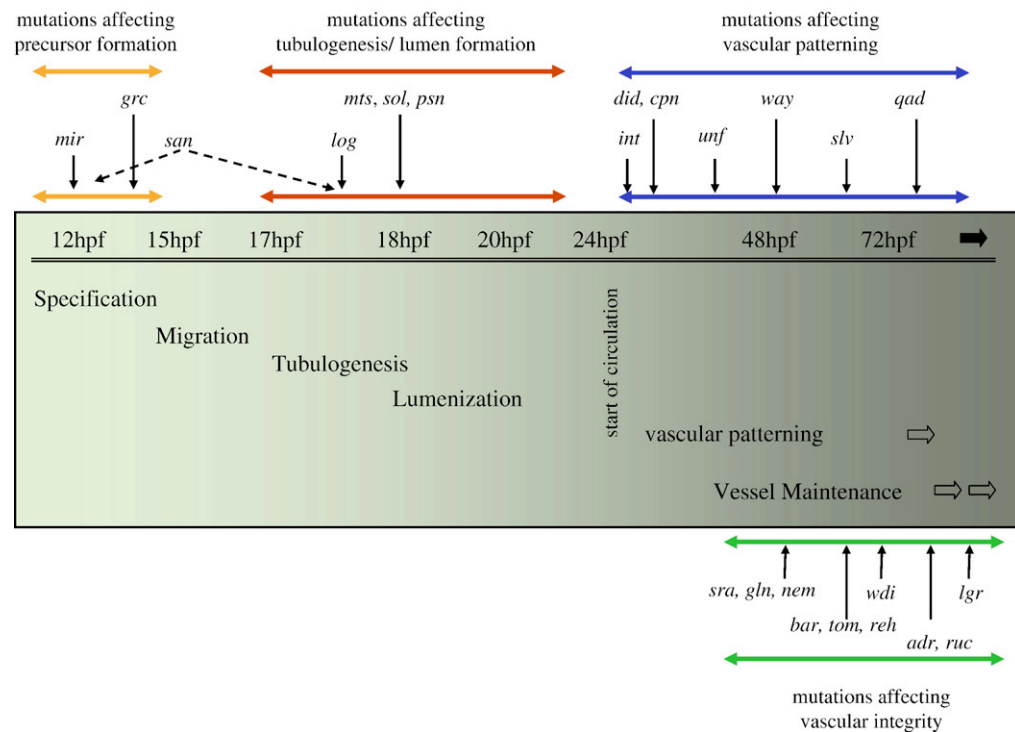


Fig. 8. Distinct groups of mutants reflect distinct developmental steps of the forming vasculature. The diagram depicts four distinct stages of vascular development, based on the mutants identified in the screen: endothelial lineage specification (Orange line), vascular tube and lumen formation (Red line), pattern formation (Blue line), and maintenance (Green line). Mutations affecting each stage are listed accordingly.

subsequent vascular patterning, and maintenance of the vasculature. The earliest event in vascular development is the specification of the angioblast, thought to occur as early as shield stage, long before gastrulation is completed. Recent data indicate that the endothelial lineage emerges from at least two distinct sources, the angioblast and the hemangioblast (Vogeli et al., 2006).

Three mutants display changes in the number of endothelial cells during early development, possibly affecting early angioblast specification. One of these, *min*<sup>s202</sup> shows defects in both endothelial and hematopoietic lineages as *clo* does (Stainier et al., 1995), suggesting that *min* might function in the hemangioblast lineage. Alternatively, *min* might function in both lineages like *scl/tal1* and *lmo2* (Liao et al., 1998; Patterson et al., 2007). Another mutation, *gnc*<sup>s635</sup>, specifically affects the endothelial lineage without affecting the hematopoietic lineage (data not included), suggesting that *gnc* may act downstream of *min* or *clo*. We observed the opposite phenotype in a new allele of *san*<sup>s234</sup>, which affects *krit1* (aka *ccm1*) (Mably et al., 2006). However, it will be interesting to determine whether *san* functions to promote the specification of nascent mesoderm into the endothelial lineage or facilitates the proliferation of angioblasts.

After their specification in the lateral plate mesoderm, endothelial cells migrate to the midline to form a vascular cord which then undergoes lumenization (Jin et al., 2005). Previously, Kamei and colleagues (2006) showed that vascular lumen formation in the zebrafish SEs occurs via vesicle fusion similar to what is observed in MDCK cells.

Several mutations appear to affect vascular tube and lumen formation without affecting early angioblast lineage specification. Further analyses will need to be performed to assess whether these mutations affect tube and lumen formation of diverse organs such as the pronephros and gut, or specifically vascular tube formation. Interestingly, three mutations, *bly*<sup>s889</sup>, *psn*<sup>s634</sup>, and *ctb*<sup>s479</sup> appear to cause dilation specifically in the PCV. Given the late onset of this phenotype and its specificity, it is tempting to speculate that differences between arterial and venous endothelial cells stabilize much later than the initial differentiation detected by molecular markers.

Subsequently, secondary vessels sprout from the lumenized axial vessels through angiogenesis (reviewed by Ema and Rossant, 2003). Increasing lines of evidence suggest that secondary vessels generated by angiogenesis follow a genetically determined pattern. Several key signaling pathways have been identified to regulate the guidance of angiogenic sprouts including Semaphorin-Plexin (Torres-Vazquez et al., 2004), Netrin-DCC (Wilson et al., 2006), and Unc5-DCC (Lu et al., 2004). These findings were instrumental in starting to understand the underlying principles of secondary vessel formation, however, most of these components specifically regulate SE sprouting. Signaling pathways that regulate sprouting of other secondary vessels, such as the brain vasculature or the subintestinal vessel, remain to be identified. We identified four mutations that affect secondary vessels other than SEs. For example, the *qad*<sup>s840</sup> mutation specifically affects vascular branching in the brain.

The cellular and molecular mechanisms that govern the maturation and maintenance of developing vessels remain largely unexplored. It is widely accepted that the major vessels become stabilized by recruiting vascular smooth muscle cells, while secondary vessels maintain their flexibility to accommodate constant demands for remodeling. Failure of these events will eventually lead to vascular regression, local hemorrhage, or global vascular cell death. We have identified 11 mutations that affect vessel maturation and maintenance. These newly identified mutants will allow one to analyze this relatively unexplored culmination of vascular development. Mutants such as *lgr*<sup>s258</sup> and *wdi*<sup>s631</sup> show a failure of vascular maintenance due to a localized regression of the vasculature. Although detailed analyses need to be performed to further delineate these phenotypes, it is possible that they are due to a failure to recruit vascular smooth muscle cells to vascular tubes. In contrast, two mutants, *bar*<sup>s847</sup> and *tom*<sup>s805</sup>, display vascular maintenance defects resulting from endothelial specific apoptosis, which will allow the investigation of this previously unexplored aspect of vascular development.

Ongoing efforts in the molecular isolation of the affected genes and further analyses of the phenotypes reported here will help understand the mechanisms by which the vascular system emerges during development and reveal the underlying genetic networks. These studies may also provide new targets for therapeutic intervention in diseases affected or caused by aberrant vasculogenesis or angiogenesis.

## Acknowledgments

We would like to thank members of the Stainier lab for invaluable discussions. Support for this research came from American Heart Association Post-doctoral fellowships (S. W. J., B. J. and E. A. O.), DFG Post-doctoral Fellowship HE4585/1-1 (W. H.), Human Frontier Science Program Organization Post-doctoral Fellowships (M. M. S., D. B. and H. V.), Canadian Institutes of Health Research Post-doctoral Fellowship (I. C. S.), Cardiovascular Research Institute NIH Training Grant positions (T. S. M., J. F., and L. A. D.), and by grants from the NIH, AHA, and Packard Foundation (D. Y. R. S.).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2007.03.526](https://doi.org/10.1016/j.ydbio.2007.03.526).

## References

- Alexander, J., Stainier, D.Y., 1999. A molecular pathway leading to endoderm formation in zebrafish. *Curr. Biol.* 9, 1147–1157.
- Amsterdam, A., Burgess, S., Golling, G., Chen, W., Sun, Z., Townsend, K., Farrington, S., Haldi, M., Hopkins, N., 1999. A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev.* 13, 2713–2724.
- Betsholtz, C., Lindblom, P., Gerhardt, H., 2005. Role of pericytes in vascular morphogenesis. *EXS* 115–125.
- Brand, M., Heisenberg, C.P., Warga, R.M., Pelegri, F., Karlstrom, R.O., Beuchle, D., Picker, A., Jiang, Y.J., Furutani-Seiki, M., van Eeden, F.J., et al., 1996. Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* 123, 129–142.
- Carmeliet, P., 2003. Angiogenesis in health and disease. *Nat. Med.* 9, 653–660.
- Carmeliet, P., 2005. Angiogenesis in life, disease and medicine. *Nature* 438, 932–936.
- Carmeliet, P., Jain, R.K., 2000. Angiogenesis in cancer and other diseases. *Nature* 407, 249–257.
- Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoek, A., Harpal, K., Eberhardt, C., et al., 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380, 435–439.
- Chan, J., Bayliss, P.E., Wood, J.M., Roberts, T.M., 2002. Dissection of angiogenic signaling in zebrafish using a chemical genetic approach. *Cancer Cell* 1, 257–267.
- Chen, J.-N., van Bebber, F., Goldstein, A.M., Serluca, F.C., Jackson, D., Childs, S., Serbedzija, G.N., Warren, K.S., Mably, J.D., Lindahl, P., et al., 2001. Genetic steps to organ laterality in zebrafish. *Comp. Funct. Genom.* 2, 60–68.
- Childs, S., Chen, J.N., Garrity, D.M., Fishman, M.C., 2002. Patterning of angiogenesis in the zebrafish embryo. *Development* 4, 973–982.
- Cleaver, O., Krieg, P.A., 1998. VEGF mediates angioblast migration during development of the dorsal aorta in *Xenopus*. *Development* 125, 3905–3914.
- Detrich III, H.W., Kieran, M.W., Chan, F.Y., Barone, L.M., Yee, K., Rundstadler, J.A., Pratt, S., Ransom, D., Zon, L.I., 1995. Intraembryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. U. S. A.* 92, 10713–10717.
- Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., et al., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123, 37–46.
- Dube, A., Akbarali, Y., Sato, T.N., Libermann, T.A., Oetgen, P., 1999. Role of the Ets transcription factors in the regulation of the vascular-specific Tie2 gene. *Circ. Res.* 84, 1177–1185.
- Ema, M., Rossant, J., 2003. Cell fate decisions in early blood vessel formation. *Trends Cardiovasc. Med.* 13, 254–259.
- Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., Moore, M.W., 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380, 439–442.
- Gupta, S., Zhu, H., Zon, L.I., Evans, T., 2006. BMP signaling restricts hemato-vascular development from lateral mesoderm during somitogenesis. *Development* 133, 2177–2187.
- Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., et al., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* 123, 1–36.
- Herzog, W., Sonntag, C., Walderich, B., Odenthal, J., Maischein, H.M., Hammerschmidt, M., 2004. Genetic analysis of adenohypophysis formation in zebrafish. *Mol. Endocrinol.* 18, 1185–1195.
- Horne-Badovinac, S., Lin, D., Waldron, S., Schwarz, M., Mbamalu, G., Pawson, T., Jan, Y., Stainier, D.Y., Abdelilah-Seyfried, S., 2001. Positional cloning of heart and soul reveals multiple roles for PKC lambda in zebrafish organogenesis. *Curr. Biol.* 11, 1492–1502.
- Isogai, S., Horiguchi, M., Weinstein, B.M., 2001. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev. Biol.* 230, 278–301.
- Jain, R.K., 2003. Molecular regulation of vessel maturation. *Nat. Med.* 9, 685–693.
- Jiang, B., Liou, G.I., Behzadian, M.A., Caldwell, R.B., 1994. Astrocytes modulate retinal vasculogenesis: effects on fibronectin expression. *J. Cell Sci.* 107, 2499–2508.
- Jin, S.W., Beis, D., Mitchell, T., Chen, J.N., Stainier, D.Y., 2005. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* 132, 5199–5209.
- Kallianpur, A.R., Jordan, J.E., Brandt, S.J., 1994. The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. *Blood* 83, 1200–1208.

- Kamei, M., Saunders, W.B., Bayless, K.J., Dye, L., Davis, G.E., Weinstein, B.M., 2006. Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* 442, 453–456.
- Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., et al., 2000. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev.* 14, 1343–1352.
- Laberge-le Couteux, S., Jung, H.H., Labauge, P., Houtteville, J.P., Lescoat, C., Cecillon, M., Marechal, E., Joutel, A., Bach, J.F., Tournier-Lasserre, E., 1999. Truncating mutations in CCM1, encoding KRIT1, cause hereditary cavernous angiomas. *Nat. Genet.* 23, 189–193.
- Larson, J.D., Wadman, S.A., Chen, E., Kerley, L., Clark, K.J., Eide, M., Lippert, S., Nasevicius, A., Ekker, S.C., Hackett, P.B., et al., 2004. Expression of VE-cadherin in zebrafish embryos: a new tool to evaluate vascular development. *Dev. Dyn.* 231, 204–213.
- Lawson, N.D., Scheer, N., Pham, V.N., Kim, C.H., Chitnis, A.B., Campos-Ortega, J.A., Weinstein, B.M., 2001. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* 128, 3675–3683.
- Lawson, N.D., Vogel, A.M., Weinstein, B.M., 2002. Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev. Cell* 3, 127–136.
- Liao, W., Bisgrove, B.W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D.J., Stainier, D.Y., 1997. The zebrafish gene *cloche* acts upstream of a *flk-1* homologue to regulate endothelial cell differentiation. *Development* 124, 381–389.
- Lindsley, R.C., Gill, J.G., Kyba, M., Murphy, T.L., Murphy, K.M., 2006. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 133, 3787–3796.
- Liao, E.C., Paw, B.H., Oates, A.C., Pratt, S.J., Postlethwait, J.H., Zon, L.I., 1998. SCL/Tal-1 transcription factor acts downstream of *cloche* to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev.* 12, 621–626.
- Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Breant, C., Claes, F., De Smet, F., Thomas, J.L., et al., 2004. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* 432, 179–186.
- Lyons, M.S., Bell, B., Stainier, D., Peters, K.G., 1998. Isolation of the zebrafish homologues for the *tie-1* and *tie-2* endothelium-specific receptor tyrosine kinases. *Dev. Dyn.* 212, 133–140.
- Mably, J.D., Chuang, L.P., Serluca, F.C., Mohideen, M.A., Chen, J.N., Fishman, M.C., 2006. Santa and valentine pattern concentric growth of cardiac myocardium in the zebrafish. *Development* 133, 3139–3146.
- Motoike, T., Loughna, S., Perens, E., Roman, B.L., Liao, W., Chau, T.C., Richardson, C.D., Kawate, T., Kuno, J., Weinstein, B.M., et al., 2000. Universal GFP reporter for the study of vascular development. *Genesis* 28, 75–81.
- Mullins, M.C., Hammerschmidt, M., Haffter, P., Nusslein-Volhard, C., 1994. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Cur. Biol.* 1, 189–202.
- Muto, A., Orger, M.B., Wehman, A.M., Smear, M.C., Kay, J.N., Page-McCaw, P.S., Gahtan, E., Xiao, T., Nevin, L.M., Gosse, N.J., et al., 2005. Forward genetic analysis of visual behavior in zebrafish. *PLoS Genet.* 1, e66.
- Ober, E.A., Verkade, H., Field, H.A., Stainier, D.Y., 2006. Mesodermal Wnt2b signalling positively regulates liver specification. *Nature* 442, 688–689.
- Oomman, S., Finckbone, V., Dertien, J., Attridge, J., Henne, W., Medina, M., Mansouri, B., Singh, H., Strahlendorf, H., Strahlendorf, J., 2004. Active caspase-3 expression during postnatal development of rat cerebellum is not systematically or consistently associated with apoptosis. *J. Comp. Neurol.* 476, 154–173.
- Parker, L.H., Schmidt, M., Jin, S.W., Gray, A.M., Beis, D., Pham, T., Frantz, G., Palmieri, S., Hillan, K., Stainier, D.Y., et al., 2004. The endothelial-cell-derived secreted factor *Egfl7* regulates vascular tube formation. *Nature* 428, 754–758.
- Park, C., Lavine, K., Mishina, Y., Deng, C.X., Ornitz, D.M., Choi, K., 2006. Bone morphogenetic protein receptor 1A signaling is dispensable for hematopoietic development but essential for vessel and atrioventricular endocardial cushion formation. *Development* 133, 3473–3484.
- Patterson, L.J., Gering, M., Patient, R., 2005. Scl is required for dorsal aorta as well as blood formation in zebrafish embryos. *Blood* 105, 3502–3511.
- Patterson, L.J., Gering, M., Eckfeldt, C.E., Green, A.R., Verfaillie, C.M., Ekker, S.C., Patient, R., 2007. The transcription factors, Scl and Lmo2, act together during development of the haemangioblast in zebrafish. *Blood* 109, 2389–2398.
- Pham, V.N., Lawson, N.D., Mugford, J.W., Dye, L., Castranova, D., Lo, B., Weinstein, B.M., 2007. Combinatorial function of ETS transcription factors in the developing vasculature. *Dev. Biol.* 303, 772–783.
- Risau, W., 1997. Mechanisms of angiogenesis. *Nature* 386, 671–674.
- Risau, W., Flamme, I., 1995. Vasculogenesis. *Annu. Rev. Cell Dev. Biol.* 11, 73–91.
- Roman, B.L., Pham, V.N., Lawson, N.D., Kulik, M., Childs, S., Lekven, A.C., Garrity, D.M., Moon, R.T., Fishman, M.C., Lechleider, R.J., et al., 2002. Disruption of *acvr1l* increases endothelial cell number in zebrafish cranial vessels. *Development* 129, 3009–3019.
- Rossant, J., Howard, L., 2002. Signaling pathways in vascular development. *Annu. Rev. Cell Dev. Biol.* 18, 541–573.
- Schier, A.F., Neuhauss, S.C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Stemple, D.L., Rangini, Z., et al., 1996. Mutations affecting the development of the embryonic zebrafish brain. *Development* 123, 165–178.
- Sehnert, A.J., Huq, A., Weinstein, B.M., Walker, C., Fishman, M., Stainier, D.Y., 2002. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat. Genet.* 31, 106–110.
- Stainier, D.Y., 2001. Zebrafish genetics and vertebrate heart formation. *Nat. Rev. Genet.* 2, 39–48.
- Stainier, D.Y., Weinstein, B.M., Detrich III, H.W., Zon, L.I., Fishman, M.C., 1995. *Cloche*, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* 121, 3141–3150.
- Stainier, D.Y., Fouquet, B., Chen, J.N., Warren, K.S., Weinstein, B.M., Meiler, S.E., Mohideen, M.A., Neuhauss, S.C., Solnica-Krezel, L., Schier, A.F., et al., 1996. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* 123, 285–292.
- Sumanas, S., Lin, S., 2006. Ets1-related protein is a key regulator of vasculogenesis in zebrafish. *PLoS Biol.* 4, e10.
- Thompson, M.A., Ransom, D.G., Pratt, S.J., MacLennan, H., Kieran, M.W., Detrich III, H.W., Vail, B., Huber, T.L., Paw, B., Brownlie, A.J., et al., 1998. The *cloche* and *spadetail* genes differentially affect hematopoiesis and vasculogenesis. *Dev. Biol.* 197, 248–269.
- Torres-Vazquez, J., Kamei, M., Weinstein, B.M., 2003. Molecular distinction between arteries and veins. *Cell Tissue Res.* 314, 43–59.
- Torres-Vazquez, J., Gitler, A.D., Fraser, S.D., Berk, J.D., Pham, V.N., Fishman, M.C., Childs, S., Epstein, J.A., Weinstein, B.M., 2004. Semaphorin-plexin signaling guides patterning of the developing vasculature. *Dev. Cell.* 7, 117–123.
- Trinh, L.A., Stainier, D.Y., 2004. Fibronectin regulates epithelial organization during myocardial migration in zebrafish. *Dev. Cell* 6, 371–382.
- Visvader, J.E., Fujiwara, Y., Orkin, S.H., 1998. Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev.* 12, 473–479.
- Vogeli, K.M., Jin, S.W., Martin, G.R., Stainier, D.Y., 2006. A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature* 443, 337–339.
- Vokes, S.A., Yatskevych, T.A., Heimark, R.L., McMahon, J., McMahon, A.P., Antin, P.B., Krieg, P.A., 2004. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. *Development* 131, 4371–4380.
- Wang, H., Charles, P.C., Wu, Y., Ren, R., Pi, X., Moser, M., Barshishat-Kupper, M., Rubin, J.S., Perou, C., Bautch, V., et al., 2006. Gene expression profile signatures indicate a role for Wnt signaling in endothelial commitment from embryonic stem cells. *Circ. Res.* 98, 1331–1339.
- Whitehead, K.J., Plummer, N.W., Adams, J.A., Marchuk, D.A., Li, D.Y., 2004. *Ccm1* is required for arterial morphogenesis: implications for the etiology of human cavernous malformations. *Development* 131, 1437–1448.
- Wijelath, E.S., Murray, J., Rahman, S., Patel, Y., Ishida, A., Strand, K., Aziz, S.,



- Cardona, C., Hammond, W.P., Savidge, G.F., et al., 2002. Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ. Res.* 91, 25–31.
- Wilson, B.D., Ii, M., Park, K.W., Suli, A., Sorensen, L.K., Larrieu-Lahargue, F., Urness, L.D., Suh, W., Asai, J., Kock, G.A., et al., 2006. Netrins promote developmental and therapeutic angiogenesis. *Science* 313, 640–644.
- You, L.R., Lin, F.J., Lee, C.T., DeMayo, F.J., Tsai, M.J., Tsai, S.Y., 2005. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* 435, 98–104.